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Growth of *Pseudomonas chloritidismutans* AW-1^T on *n*-alkanes with chlorate as electron acceptor

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Abstract Microbial (per)chlorate reduction is a unique process in which molecular oxygen is formed during the dismutation of chlorite. The oxygen thus formed may be used to degrade hydrocarbons by means of oxygenases under seemingly anoxic conditions. Up to now, no bacterium has been described that grows on aliphatic hydrocarbons with chlorate. Here, we report that *Pseudomonas chloritidismutans* AW-1^T grows on *n*-alkanes (ranging from C7 until C12) with chlorate as electron acceptor. Strain AW-1^T also grows on the intermediates of the presumed *n*-alkane degradation pathway. The specific growth rates on *n*-decane and chlorate and *n*-decane and oxygen were 0.5±0.1 and 0.4±0.02 day^{-1}, respectively. The key enzymes chlorate reductase and chlorite dismutase were assayed and found to be present. The oxygen-dependent alkane oxidation was demonstrated in whole-cell suspensions. The strain degrades *n*-alkanes with oxygen and chlorate but not with nitrate, thus suggesting that the strain employs oxygenase-dependent pathways for the breakdown of *n*-alkanes.

Keywords *n*-Alkane oxidation · Chlorate reduction · *Pseudomonas chloritidismutans* AW-1^T

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Introduction

Petroleum, a complex mixture of aromatic and aliphatic hydrocarbons, is one of the most common environmental contaminants. On average, saturated and aromatic hydrocarbons together make 80% of the oil constituents (Widdel and Rabus 2001). Since the saturated hydrocarbon fraction is the most abundant in crude oil, its biodegradation is quantitatively most important in oil bioremediation (Head et al. 2006). *n*-Alkanes are relatively stable due to lack of functional groups, presence of only sigma bonds, nonpolar nature, and low solubility in water.

Aerobic microbial degradation of *n*-alkanes is known since almost a century (Söhngen 1913), and the mechanisms of degradation, with the enzymes and genes involved, are rather well understood (Berthe-Corti and Fetzner 2002; Head et al. 2006; van Beilen and Funhoff 2007; Wentzel et al. 2007). During aerobic degradation, molecular oxygen acts as a cosubstrate and as a terminal electron acceptor (Berthe-Corti and Fetzner 2002; Chayabutra and Ju 2000). Oxygenases incorporate molecular oxygen into the *n*-alkanes to form the corresponding alcohols, which are further degraded by beta-oxidation (Wentzel et al. 2007). Since intermediates do not accumulate, the initial step of oxygen incorporation seems to be the rate-limiting step (Chayabutra and Ju 2000).

Insight into anaerobic degradation of *n*-alkanes is limited. The first step of anaerobic degradation of *n*-alkanes is thermodynamically difficult and has been proposed to occur in the sulfate-reducing bacterium strain Hxd3 via carboxylation (So et al. 2003). Molecular evidence for a mechanism of *n*-alkane activation through fumarate addition was obtained recently (Callaghan et al. 2008; Grundmann et al. 2008). Anaerobic degradation of *n*-alkanes is slow compared to aerobic degradation (Wentzel et al. 2007), and

only a few denitrifying and sulfate-reducing bacteria have been isolated (Ehrenreich et al. 2000; So and Young 1999).

Microbial (per)chlorate reduction is a process that yields molecular oxygen, a property that has application possibilities in the bioremediation of polluted anoxic soils (Coates et al. 1998; Tan et al. 2006; Weelink et al. 2008). During chlorate reduction, chlorate (ClO_3^-) is reduced to chlorite (ClO_2^-) by the enzyme chlorate reductase. Chlorite is then split into Cl^- and O_2 by chlorite dismutase (Rikken et al. 1996; Wolterink et al. 2002). The oxygen released during chlorate reduction might be used to degrade *n*-alkanes by oxygenases.

Here, we report the finding that *Pseudomonas chloritidismutans* AW-1^T, a chlorate-reducing bacterium, that was previously isolated in our laboratory with acetate as carbon and energy source is able to grow on *n*-decane with oxygen or chlorate as electron acceptor. This finding suggests that an additional function of chlorite dismutation is to generate molecular oxygen to perform oxygenase-dependent reactions to support growth on *n*-alkanes.

Materials and methods

Inoculum, media, cultivation, and counting

P. chloritidismutans strain AW-1^T (DSM 13592^T) was isolated in our laboratory (Wolterink et al. 2002) and was kindly provided by Servé Kengen. For experiments with nitrate, it was adapted to nitrate by repeated subculturing on acetate and nitrate, while gradually decreasing the oxygen concentration according to Cladera et al. (2006).

The medium for *P. chloritidismutans* strain AW-1^T was based on the medium described by Dorn et al. (1974). The composition of the medium (in grams per liter of anaerobic demineralized water) was as follows: $Na_2HPO_4{\cdot}2H_2O$, 3.48; KH_2PO_4 1; resazurin, 0.005; $CaCl_2$, 0.009; ammonium iron (III) citrate, 0.01; NH_4SO_4, 1; $MgSO_4{\cdot}7H_2O$, 0.04. Vitamins and trace elements were added as described by Holliger et al. (1993) supplemented with Na_2SeO_3, 0.06 and $NaWO_4{\cdot}2H_2O$ 0.0184. The pH of the medium was 7.3.

P. chloritidismutans strain AW-1^T was cultivated in 120-ml flasks containing 40 ml of medium at 30°C. The medium was made in anaerobic water and dispensed in the flasks under continuous flushing with nitrogen. The bottles were closed with viton stoppers (Maag Technik, Dübendorf, Switzerland) and aluminum crimp caps, and the head space was replaced by N_2 gas (140 kPa). All solutions that were added to the medium were made anaerobic and autoclaved at 121°C for 20 min. The $CaCl_2$ was autoclaved separately to avoid precipitation and added aseptically to the already autoclaved salt solution. Vitamins and trace elements were filter-sterilized. Chlorate and nitrate were supplied from a 0.4-M stock solution to get a final concentration of 10 mM. Pure oxygen was added from a sterilized gas stock. To prepare a stock solution of *n*-decane, a flask was made anaerobic by flushing with N_2 and then autoclaved. Ten microliters (1.28 mM final concentration) of sterile 99% pure *n*-decane (Merck) was added to the medium through a 0.2-μm membrane filter. For mass balance analyses, 1 mM of *n*-decane was added from a 50-mM stock solution of *n*-decane in acetone. The inoculum size for cultivation was 10% (*v*/*v*). Other *n*-alkanes tested were *n*-propane, *n*-butane, *n*-pentane, *n*-hexane, *n*-heptane, *n*-octane, *n*-nonane, *n*-undecane, *n*-dodecane, *n*-tetradecane, and *n*-hexadecane. 1-Decanol was added from an anaerobic filter-sterilized solution, while sodium decanoate was added from an anaerobic autoclaved 0.4-M stock solution.

Cell numbers were enumerated by phase-contrast microscopy using a Bürker-Türk counting chamber at ×1,000 magnification.

Three aerobic alkane-utilizing bacteria, *Alcanivorax borkumensis* SK2 (DSM 11573), *Acinetobacter* sp. strain (DSM 17874), and *Acinetobacter baylyi* (DSM 14961), and the non-alkane-degrading *Pseudomonas putida* KT2440 (DSM 6125) were obtained from the DSMZ, Braunschweig, Germany.

Preparation of cell-free extracts

Cell-free extracts of strain AW-1^T, grown in anaerobic medium with *n*-decane as sole carbon and energy source and chlorate as electron acceptor, were prepared anaerobically as previously described by Wolterink et al. (2002). The only modification was the centrifugation of whole cells at 13,000 rpm for 10 min at 4°C. Cell-free extracts were stored under a N_2 gas phase at 4°C in 12-ml serum vials.

The protein content of the cell-free extract fraction was determined according to the method of Bradford (1976) with bovine serum albumin as standard.

Enzyme activity measurements

Chlorate reductase and chlorite dismutase activities were determined with cell-free extracts. Chlorate reductase activity was determined spectrophotometrically as described by Kengen et al. (1999), by monitoring the oxidation of reduced methyl viologen at 578 nm and 30°C. One unit (U) of enzyme activity is defined as the amount of enzyme required to convert 1 μmol of chlorate per minute.

Chlorite dismutase activity was determined by measuring oxygen production with a Clark-type oxygen electrode (Yellow Spring Instruments, Yellow Springs, OH, USA) as described by Wolterink et al. (2002). One unit of activity is defined as the amount of enzyme required to convert 1 μmol of chlorite per minute.

Alkane oxidation activity was determined by measuring the decrease in *n*-decane concentration in time by gas chromatography with whole-cell suspensions. Cells were harvested by centrifugation at 13,000 rpm for 10 min at 4°C. Cells were washed and suspended in the buffer and then starved for 2 days to decrease the endogenous activity. After starvation, cells were suspended in a 15-mM phosphate buffer containing 2.5 mM *n*-decane added from a 50-mM stock solution in acetone. The reaction mixture was placed in a shaker set at 180 rpm at 30°C. Two-milliliter samples were taken in duplicate periodically after 0, 5, 10, 15, and 30 min. The *n*-decane was extracted as described by Staijen et al. (2000). Cells treated for 10 min at 100°C were used as controls. One unit of activity is defined as the amount of enzyme required to convert 1 μmol of *n*-decane per minute. Starved cells, resuspended in the anoxic buffer, flushed with nitrogen, and reduced with 3 mM cysteine, were used as anaerobic control.

Alcohol dehydrogenase activity was determined spectrophotometrically in the reductive direction at 30°C using a spectrophotometer (U-2010, Hitachi). The activities were assayed in 1-ml reaction mixtures containing 15-mM sodium phosphate buffer with 0.3 mM NADH and 0.5 mM aldehyde. The decrease in A_{340} was monitored to assess the activity. One unit of enzyme activity is defined as the amount of enzyme required to oxidize 1 μmol of NADH. We also tried to determine the alcohol oxygenase activity by the above-mentioned spectrophotometric method using NADH and air-flushed reaction mixtures.

Analytical techniques

Chlorate, chloride, nitrate, and nitrite were measured, as described by Scholten and Stams (1995) after separation on a Dionex column (Ionpac AS9-SC; Breda, The Netherlands), with a conductivity detector. Potassium fluoride (2 mM) was used as internal standard.

Oxygen was analyzed by gas chromatography with a GC-14B apparatus (Shimadzu, Kyoto, Japan) equipped with a packed column (Molsieve 13× 60/80 mesh, 2-m length, 2.4-mm internal diameter; Varian, Middelburg, The Netherlands) and a thermal conductivity detector. The oven temperature used was 100°C and the injector and detector temperatures were 90°C and 150°C, respectively. Argon was used as the carrier gas at a flow rate of 30 ml min^{-1}.

CO_2 was analyzed by gas chromatography on a Chrompack CP9001 gas chromatograph fitted with a thermal conductivity detector (Henstra and Stams 2004). pH was measured by a glass microelectrode connected to a pH meter (Radiometer, Copenhagen). Total amount of bicarbonate present inside the flask was calculated by using the Henderson-Hesselbach equation. At 30°C, the α and pK' are 0.665 and 6.348, respectively (Breznak and Costilow 1994).

n-Decane was extracted from 40-ml culture with 20 ml of *n*-hexane by shaking for 3 h and then separating the two phases through a separating funnel. *n*-Decane was analyzed in the hexane phase after adding octane as internal standard. One microliter was injected with a CP9010 autosampler in a CP9001 gas chromatograph (Chrompack), equipped with flame ionization detector and having a Chrompack Sil 5 CB capillary column (length, 25 m; diameter, 0.32 mm; *df*, 1.2 um) with nitrogen, 50-kPa inlet pressure, as carrier gas. The temperature of the injector, column, and the detector was 250°C, 100°C, and 300°C, respectively.

Detection of alkane oxygenase genes

For the detection of putative genes encoding alkane oxygenase, the genomic DNA was extracted from cultures of strain AW-1^T grown on *n*-decane and chlorate using a FastDNA SPIN kit for Soil (Qbiogene). The extracted DNA was precipitated with isopropanol and vacuum-dried. The primers developed by Whyte et al. (2002), Heiss-Blanquet et al. (2005), Kloos et al. (2006), and van Beilen et al. (2006) were used for the detection of the alkane oxygenases *alkB*, *alkM*, *almA*, and *P450*. In addition, we developed some degenerated primers targeting *alkB*, *almA*, and Acyl-CoA dehydrogenase. Polymerase chain reaction (PCR) was carried out under the following conditions: final volume of 50 μl of 1× PCR buffer (Promega) supplemented with $MgCl_2$ (2.5 mM), 200 μM dNTPs, 0.5 μM of each primer, and 0.3 U/μl Taq DNA Polymerase (Promega). The amplification conditions used were as follows: an initial denaturation step at 94°C for 3 min, 35 times of a three-step cycle of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min, and a final elongation step of 72°C for 8 min. Polymerization reactions were stopped by cooling the samples at 4°C. In addition, a gradient PCR using a temperature gradient of 40–65°C was done with the *alkB* and *Cyt P450* primers used above and also with the primers designed by Smits et al. (1999, 2002) and Kohno et al. (2002) and the degenerate primers used by Kubota et al. (2005). Sequences of the primers used in this study are given in Table 1. The positive controls of amplification were obtained in reactions with primers targeting *alkB* and CYP153 genes using genomic DNA extracted from *A. borkumensis* SK2, in reactions with primers targeting *almA*, with *Acinetobacter* sp. strain DSM 17874 genomic DNA, and with *A. baylyi* DSM 14961 in reactions targeting *alkM*.

Nucleotide sequence accession numbers

The DNA sequence of a putative acyl-CoA dehydrogenase gene of *P. chloritidismutans* AW-1^T was deposited in the GenBank/European Molecular Biology Laboratory/DNA Data Bank of Japan under accession number FJ477383.

Table 1 Different primer sets used in this study targeting conserved regions of *alkB*, *almA*, *cytochrome P450 subfamily CYP153*, and *acyl-CoA dehydrogenase* genes

Primer name	Sequence (5′⟶3′)	Reference
alkB		
TS2Sf	AAYAGAGCTCAYGARYTRGGTCAYAAG	Smits et al. 1999
TS2Smodf	AAYAGAGCTCAYGARITIGGICAYAAR	
TS2Smod2f	AAYAGAGCTCAYGARITITCICAYAAR	
DEG1RE	GTRAGICTRGTRGTRCGCTTAAGGTG	
DEG1RE2	GTRTCRCTRGTRGTRCGCTTAAGGTG	
alkMUp	CGGGGTAAGCATGAATAGCT	Tani et al. 2001
alkMDn	CGTACAGCTACTTGGTGGAC	
Alk-1F	CATAATAAAGGGCATCACCGT	Kohno et al. 2002
Alk -1R	GATTTCATTCTCGAAACTCCAAAC	
Alk-3F	CCGTAGTGCTCGACGTAGTT	
Alk-3R	CAGGCGTTCTTCGGGTTGCGCTGCTCGA	
AlkBpaFwd	AACTGGAATTCACGATGTTTGA	Smits et al. 2002
AlkBpaRv2	CTGCCCGAAGCTTGAGCTAT	
AlkBpaBfw	GGAGAATTCTCAGACAATCT	
AlkBpaBrv	GAGGCGAATCTAGAAAAAACTG	
B5 -Eco	GGAGAATTCCAAATGCTTGAG	
B3-Hind	TTTGTGAAAGCTTTCAACGCC	
Pp alkB-F	TGGCCGGCTACTCCGATGATCGGAATCTGG	Whyte et al. 2002
Pp alkB-R	CGCGTGGTGATCCGAGTGCCGCTGAAGGTG	
Rhose	ACGGSCAYTTCTACRTCG	Heiss-Blanquet et al. 2005
Rhoas	CCGTARTGYTCGAGRTAG	
Pseuse1	GARCATAATAARGGBCATC	
Pseuas1	AGCARWCCGTARTGYTCA	
Pseuse2	AYGTSCGYGGCCACCATGT	
Pseuas2	CGACGTAGTTGAYGAYYTCC	
Acinse	ACWCCTGAAGATCCRGCWTC	
Acinas	TRTTCCATCTAGCTCWGGC	
alkB-1f	AAYACNGCNCAYGARCTNGGNCAYAA	Kloos et al. 2006
alkB-1r	GCRTGRTGRTCNGARTGNCGYTG	
alkBF	GSNCAYGARYTSRKBCAYAA	This study
alkBR	GCRTGRTGRTCNSWRTGNCGYTG	
Cytochrome P450 subfamily CYP153		
CF	ATGTTYATHGCNATGGAYCCNC	Kubota et al. 2005
CR	NARNCKRTTNCCCATRCANCKRTG	
P450fw1	GTSGGCGGCAACGACACSAC	van Beilen et al. 2006
P450rv3	GCASCGGTGGATGCCGAAGCCRAA	
almA		
almA-F1	CCBGGBATYCGBTCNGAYTCNGAYATGT	This study
almA-R1	GGHGADCGYTGYARCATVGTNACGTNACBATGYTRCARCGHTCDCC	
almA-R2	CANAVVCGYTSRTCCCANGGVTTRTATAYAABCCNTGGGAYSARCGBBTNTG	
Acyl-CoA dehydrogenase		
Acyl-F1	GGYTCNATYGARCABAARATGGG	This study
Acyl-R1	CCCCAYTCRCGRATRWARCCRTGVCCRCCRAA	
Acyl-R2	TGRAYRCCRTTRGTRCCTTCRTARAT	

Results

n-Alkane degradation

P. chloritidismutans AW-1^T uses *n*-decane as a sole source of carbon and energy. Growth on *n*-decane and chlorate was indicated by the increase in optical density (Fig. 1a). An OD of 0.34 corresponds to a bacterial count of 1.31×10^9. Growth followed *n*-decane degradation as indicated by CO_2 formation, chlorate reduction, and chloride production (Fig. 1a). No growth was observed in controls without inoculum or without *n*-decane or controls without chlorate (results not shown). The specific growth rate on *n*-decane and chlorate was 0.5±0.1 day^{-1} (doubling time 1.4± 0.2 days). After 7 days, 87% of the 1 mM of the added *n*-decane was oxidized. The oxidation of 1 mM of *n*-decane led to a reduction of 9.2±0.7 mM of chlorate and yielded 7.7±0.6 mM of bicarbonate and 8.3±0.8 mM of chloride. The balance fits relatively well with the theoretical stoichiometry of complete oxidation of *n*-decane coupled to chlorate reduction:

$$C_{10}H_{22} + 10.33ClO_3^- \rightarrow 10HCO_3^- + 10.33Cl^- + H_2O + 10H^+ \quad (1)$$

The bacterium also grows aerobically on *n*-decane (Fig. 1b). The specific growth rate on *n*-decane and molecular oxygen was 0.4±0.02 day^{-1} (doubling time 1.7± 0.1). Growth and CO_2 production were not observed in the presence of *n*-decane and nitrate, using *P. chloritidismutans* adapted to growth on nitrate and acetate (results not shown).

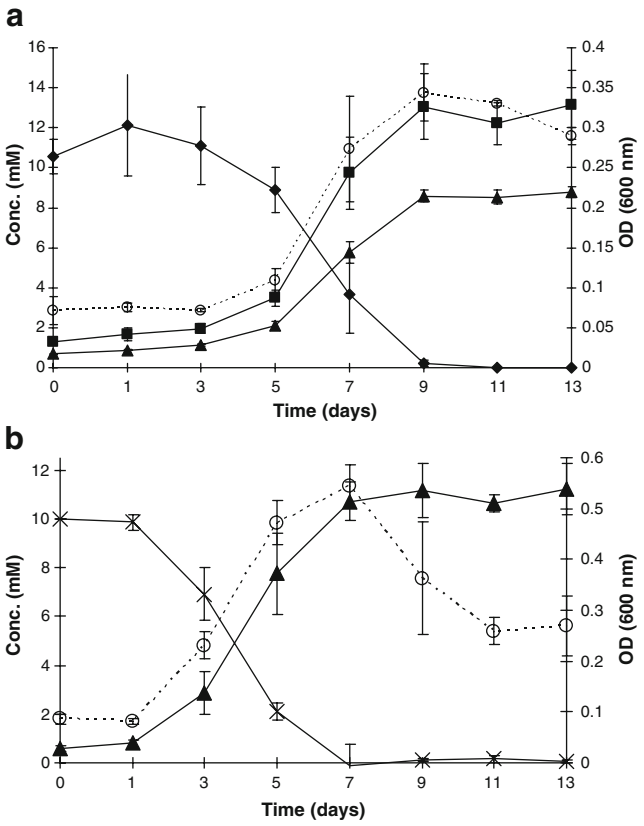

Fig. 1 Growth of strain AW-1^T **a** with decane and chlorate and **b** with decane and oxygen. Values are means of three replicates. The *bars* represent standard deviation. *Dotted line* with *open circles* represents the OD at 600 nm. The *continuous lines* represent (*diamonds*) chlorate utilized; (× *marks*) O_2 utilization; (*squares*) chloride produced, and (*triangles*) bicarbonate formed

Other substrates utilized

Apart from *n*-decane, other *n*-alkanes were also screened as possible substrates with chlorate as electron acceptor. Strain AW-1^T grew with C7 to C12 n-alkanes but not with smaller *n*-alkanes. It grew equally well on odd and even chain *n*-alkanes. Strain AW-1^T grew equally well on C8 until C11, while growth on C7 and C12 was slower.

Strain AW-1^T also grew on the possible intermediates of the aerobic *n*-alkane degradation pathway, namely, 1-decanol and decanoate. Table 2 shows the amount of bicarbonate formed with various substrates using different electron acceptors. With *n*-decane and 1-decanol as substrates, bicarbonate was formed with chlorate and oxygen but not with nitrate as electron acceptor. With decanoate and nitrate, bicarbonate was also formed.

Enzyme assays

Extracts of cells grown on *n*-decane and chlorate and on acetate and chlorate showed chlorate reductase and chlorite dismutase activity (Table 3). The specific chlorite dismutase activity is dependent on the amount of cell extract and the chlorite concentration (Mehboob et al. 2009). The chlorite dismutase presented in Table 3 is the activity under optimal conditions.

Alkane oxidation activity could be measured with starved cells grown on *n*-decane and chlorate. A relatively small amount of activity was also observed with starved whole cells grown on acetate and chlorate (Table 3). No alkane oxidation was observed with the anoxic control. Cell-free extract of alkane-grown cells did not show alcohol oxygenase activity, but we found an activity of 0.06 U/mg of protein of NAD$^+$-dependent decanol dehydrogenase. This was quite surprising as during the growth experiment strain AW-1^T was unable to grow with decanol and nitrate. Our strain is known to grow with ethanol and chlorate (Wolterink et al. 2002). We checked and found that strain

Table 2 Formation of bicarbonate (in millimolar) by strain AW-1^T during growth on different substrates. Values for decane are after 9 days, while for decanol and decanoate the samples were analyzed after 7 days

	Electron acceptor		
Substrate	O_2	ClO_3^-	NO_3^-
n-Decane	10.6±1.1	7.9±0.3	0.7±0.2
1-Decanol	9.8±1.3	9.8±0.3	0.2±0.1
n-Decanoate	8.3±0.4	8.6±0.4	8.0±0.9

AW-1^T is able to grow with ethanol using oxygen, chlorate, and nitrate as electron acceptors. Even ethanol- and nitrate-adapted cells could not grow on the decanol and nitrate. Cell-free extract of decane-grown culture showed twofold higher activity of 0.15 U/mg of protein with acetaldehyde.

Detection of alkane oxygenase genes

Various primers at different annealing temperatures were used to detect the following alkane oxygenase genes: *alkB*, *alkM*, *almA*, and cytochrome P450 subfamily CYP153. Though we got the expected results in positive controls, with the available specific primers sets, we were not able to detect any kind of known alkane oxygenase genes in our strains. We were able to amplify a sequence which was 51% and 57% similar to two acyl-CoA dehydrogenases involved in the degradation of *n*-alkane in *Acinetobacter* strain M-1 (Tani et al. 2002).

Discussion

P. chloritidismutans AW-1^T is a gram-negative, facultative, anaerobic, and chlorate-reducing bacterium, which has been isolated on acetate and chlorate in our laboratory (Wolterink et al. 2002). We tested its ability to grow on *n*-alkanes and found that strain AW-1^T grows on *n*-alkanes with oxygen and chlorate as electron acceptor. Many pseudomonades have the ability to grow aerobically on *n*-alkanes (Söhngen 1913; Wentzel et al. 2007), but strain AW-1^T is the only known bacterium that grows on *n*-alkanes by supplying molecular oxygen formed by chlorite dismutation. The doubling time with *n*-decane and chlorate is 1.4±0.2 days. Except for strain HxN1, which has a doubling time of 11 h (Ehrenreich et al. 2000), all other anaerobic *n*-alkane degraders grow slower, e.g., strains Hxd3 and Pnd3 have doubling times of 9 days (Aeckersberg et al. 1998) and strain AK-01 has a doubling time of 3 days (So and Young 1999). In contrast, doubling times of aerobic alkane-degrading bacteria are approximately 1 h for *Pseudomonas aeruginosa* (Ertola et al. 1965) and 5 h for *Rhodococcus* species (Bredholt et al. 1998).

Strain AW-1^T grows on *n*-decane with chlorate and oxygen but not with nitrate, suggesting the involvement of oxygenases. Oxygen is incorporated in *n*-decane through an oxygenase to form decanol. When chlorate is used as electron acceptor, oxygen is formed by dismutation of chlorite. This is supported by the similar specific growth rates on *n*-decane with oxygen (0.5±0.1 day^{-1}) or chlorate (0.4±0.02 day^{-1}) as electron acceptor. Strain AW-1^T also grows on possible aerobic intermediates, like 1-decanol and decanoate with oxygen and chlorate as electron acceptor. It was unable to utilize 1-decanol when nitrate was used as electron acceptor. This also suggests that, in the conversion of decanol, an oxygenase as found by Buhler et al. (2000) and Katopodis et al. (1984) is involved. Growth on decanoate with nitrate suggests that no oxygenases are required for decanoate degradation. Growth of strain AW-1^T with oxygen or chlorate was observed to be the fastest on decanoate followed by decanol and then *n*-decane.

We faced a problem in detecting alkane oxygenase activity with cell-free extracts. This has also been observed by others (Katopodis et al. 1984; Tani et al. 2001) and was attributed to the poor solubility of the substrate (Smits et al. 2002; Tani et al. 2001), the instable nature of the alkane oxygenase complex (Katopodis et al. 1984; McKenna and Coon 1970; Ruettinger et al. 1974), and the involvement of

Table 3 Activities of chlorate reductase, chlorite dismutase, and alkane oxygenase of strain AW-1^T grown on acetate and chlorate or on *n*-decane and chlorate

	Specific enzyme activity (units per milligram of protein)	
	Acetate+ClO_3^-	*n*-Decane+ClO_3^-
Chlorate reductase	11.4±0.3	26.6±1
Chlorite dismutase	7.7±1.4	2.8±0.7
Alkane oxygenase[a]	26±9	93±31

[a] The alkane oxidation activity was measured with whole cells and is average of the activities at four different time points, i.e., after 5, 10, 15, and 30 min

unknown factors (Tani et al. 2001), like some unique electron transfer proteins (van Beilen et al. 2006). However, alkane oxidation activity could be demonstrated with whole cells grown on *n*-decane and chlorate. Almost 3.5-fold more activity was observed with the cells grown on decane and chlorate as compared with the cells grown on acetate and chlorate showing the induction of alkane oxygenase when grown on *n*-decane.

We were unable to detect an alcohol oxygenase in decane-grown cell-free extracts. However, we found an alcohol dehydrogenase that has more than twofold higher activity for acetaldehyde than for decanal. Since the strain AW-1^T grows with ethanol with oxygen, chlorate, and nitrate but is unable to grow with decanol and nitrate, it is unlikely that this alcohol dehydrogenase is involved in long-chain alcohol oxidation. Instead, the detected alcohol dehydrogenase only seems to be involved in growth with short-chain alcohols.

We were unable to amplify an alkane oxygenase gene from our strain with new and known primers designed to detect different classes of alkane oxygenases. However, we amplified a sequence, similar to an acyl-CoA dehydrogenase from strain AW-1^T. This acyl-CoA dehydrogenase enzyme has been reported to be involved in *n*-alkane degradation in *Acinetobacter* strain M-1 (Tani et al. 2002). The same group proposed that a dioxygenase is involved in the initial oxidation (Finnerty pathway) of *n*-alkanes (Maeng et al. 1996). However, we observed that the N-terminal sequence of this dioxygenase and the above-mentioned acyl-CoA dehydrogenase is similar. This sequence seems highly conserved in all *Pseudomonas* genomes. Therefore, we also did a growth test on *n*-alkanes as carbon and energy source with *Pseudomonas* sp. KT2440, for which the genome sequence is available. The genome contains acyl-CoA dehydrogenase genes, but evidence for the presence of a conventional alkane hydroxylase system is lacking. No obvious aerobic growth of this strain on *n*-alkanes was found. Hence, we assume that the acyl-CoA dehydrogenase is not involved in the first step of activation of *n*-alkane, as reported by Maeng et al. (1996), but is important in a later reaction step of *n*-alkane degradation.

A reason why we were not able to detect alkane oxygenase genes could be that the alkane oxygenases have a very high sequence diversity (i.e., the protein sequence similarity between reported *alkB* types can be as low as 35%), especially among the *Pseudomonas* group. The *Pseudomonas* alkane oxygenases are as distantly related to each other as to the alkane oxygenases from phylogenetically unrelated bacteria (Smits et al. 2002). This may have led to similar false-negative results as reported by others (Chandler and Brockman 1996; Heiss-Blanquet et al. 2005; van Beilen et al. 2006). It may also be that a novel type of alkane oxygenase is involved in this process, of

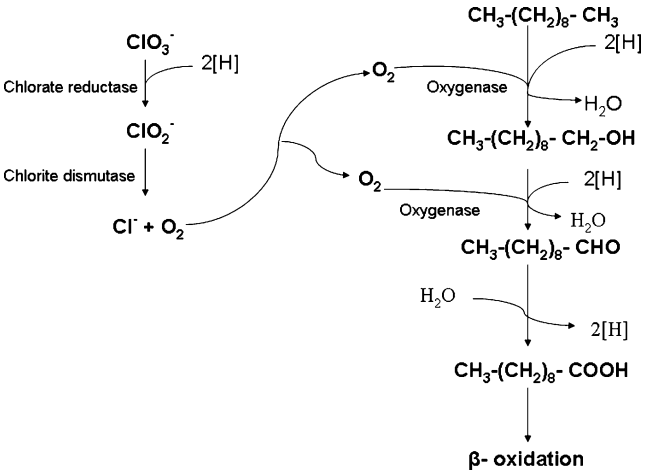

Fig. 2 Hypothetical pathway of degradation of *n*-decane coupled to chlorate reduction. Oxygen released from chlorite dismutation is used by a presumed oxygenase to incorporate in the *n*-alkane molecule to form an alcohol and later on an aldehyde. Further degradation may occur in the absence of oxygen

which the genes are not known yet and which may be specific for *n*-alkane degradation at low oxygen concentrations, as is evidently the case for growth under chlorate-reducing conditions. The extent of diversity of alkane oxygenases became apparent in recent research by Kuhn et al. (2009). They found that only one out of the 76 clones of the putative *alk* genes had a significant sequence similarity with previously known *alk* genes.

Based on all the physiological features, enzyme measurements, and the amplification of an acyl-CoA dehydrogenase gene, we propose a hypothetical *n*-alkane (C7–C12) degradation pathway as depicted for *n*-decane in Fig. 2. We suggest that oxygen formed in the dismutation of chlorite is used to convert *n*-decane to decanol and decanol to decanal by means of oxygenases. Decanal is further oxidized to decanoate, which upon activation is degraded by β-oxidation.

This is the first report of the degradation of aliphatic hydrocarbons with chlorate, both as electron acceptor and as source of oxygen needed for the oxygenase activity. The degradation of aromatic hydrocarbons with chlorate has recently been described (Tan et al. 2006; Weelink et al. 2008). This study adds to the possibility to apply microorganisms with oxygenase-dependent pathways for the bioremediation of anoxic soils polluted with compounds that are difficult to degrade in the absence of molecular oxygen.

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